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STUDIES UPON CALCAREOUS DEGENERATION.

I.

THE PROCESS OF PATHOLOGICAL CALCIFICATION.

BY

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PLATES XXX AND XXXI.

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I. THE PROCESS OF PATHOLOGICAL CALCIFICATION.

BY OSKAR KLOTZ,

*Demonstrator and Late Governors' Fellow in Pathology, McGill University,
Montreal.*

PLATES XXX AND XXXI.

INTRODUCTION.

METHODS.

Staining methods.

Chemical methods.

THE PROCESS OF DEPOSIT OF CALCIUM SALTS IN BONE.

THE PROCESS OF DEPOSIT OF CALCIUM SALTS UNDER PATHOLOGICAL CONDITIONS.

In the arterial wall.

In chronic inflammation of the pleura.

In calcified fibromata.

In the walls of ovarian and broad-ligament cysts.

In tubercular nodules.

In pancreatic fat necrosis.

ON THE PRESENCE OF SOAPS IN THE ORGANISM UNDER PHYSIOLOGICAL CONDITIONS.

ON THE PRESENCE OF SOAPS IN THE ORGANISM UNDER PATHOLOGICAL CONDITIONS.

The isolation of soaps from pus.

ON THE EXISTENCE OF A COMPOUND OF SOAPS WITH ALBUMEN.

ON THE EXPERIMENTAL PRODUCTION OF CALCIFICATION.

In capsules containing fats and fatty acids.

In the kidney.

In muscle.

In fat necrosis.

ON THE SOURCE OF THE FAT IN THE DEGENERATED CELLS.

CONCLUSIONS.

BIBLIOGRAPHY.

INTRODUCTION.

It is a well known fact and a not uncommon occurrence that the interest in certain subjects or lines of thought fluctuates widely; we are ever too ready, when we think we have obtained an explanation of a certain phenomenon, to take much for granted and allow our enthusiasm to lapse. Such has been the case in the study of pathological calcification, which has gone through several periods of new theories and rest.

¹ This study was begun while the writer was Governors' Fellow in Pathology of McGill University, and has been continued and completed under a grant from the Rockefeller Institute for Medical Research.

Notwithstanding these theories, in reviewing the literature concerning calcareous degeneration or calcification, one is struck with the dearth of actual facts that are set forth upon which those theories are based. In truth the labor that has been spent in the actual study of the *process* has not been extensive and it is confined to a few papers in the last decade. There have been many who have reported the occurrence of calcareous degeneration of diverse tissues and have even gone minutely into the chemical nature of the organic salts present, but here the study has ended. Regarding the existence of pathological calcification we have abundant data, as to the process of calcification we know little.

Indeed, calcification is one of the commonest pathological conditions noted by the older writers, especially that occurring in the arteries and in fibromata of the uterus. Not infrequently the change in the arteries is spoken of as appearing "partly fatty and partly earthy." No doubt reference was made to the yellow atheromatous areas overlying calcareous plaques in the arterial wall. Not until Virchow in 1858 asserted that inorganic salts were deposited in supersaturated solutions of the same and that the deposit of calcium was of this nature, was there any discussion on the subject. Virchow held that calcareous deposits were chiefly found in old age and in diseases causing wasting of the bony framework, and that in this state calcium was dissolved out of the osseous system and on account of the too concentrated solution of calcium salts in the blood these were laid down elsewhere as metastatic deposits. In this way he explained the common occurrence in old people of calcareous plaques in the arteries.

Some years later von Recklinghausen reported the studies he had made upon the degenerations. He pointed out that calcium deposits result partly in tissues which are well supplied with tissue fluid, but also in tissues poorly supplied. When blood or tissue fluids are saturated with calcium salts, due to repeated breaking down of bony structures, he noted a secondary incrustation of the lung and kidney tissue. But there were other cases in which, as in fibroids and phleboliths, calcification proceeded under conditions of normal calcium content of the

blood. Von Recklinghausen looked on the processes of ossification and petrification as similar chemically, though differing biologically, in that the former took place in active and living cells, the latter in degenerating or dead tissues. He held that the factors governing calcification were: (i) the separation of sparingly soluble lime salts, (ii) the existence of these substances in excess in the fluids, and (iii) the presence of a foreign body which acts as a nucleus.

These factors would be of weight in considering fluids outside the body but not within. The lime salts in the tissue fluids are not found in excess during calcification and the common salts of lime found in the tissue fluids are very soluble. The deposit of calcareous plaques in the serous membranes and in the arterial walls he considered under ossification and as distinct from calcification.

Lancereaux's views were very similar to those of von Recklinghausen, though he attributed the supersaturation of lime salts in the blood to a hindrance of the excretion of these salts by the urine, and found them accumulated in the pyramids of the kidney and the mucosa of the stomach and intestines.

Thus it was that up to the time of Litten's ingenious experiments (1881), the theory of the excess of lime salts in the blood was accepted as explaining calcareous degeneration. No note was taken of the predilection of certain tissues for this deposit of lime, except that dead or dying tissues were regarded as a nucleus about which the carbonate and phosphate of calcium would crystallize. With Litten's studies—which will be discussed more fully later—a new theory was raised which superseded that inaugurated by Virchow, and up to the present time his has been the explanation of calcification accepted in the textbooks. After the appearance of Litten's work many followed in his footsteps, the majority supporting his views, although some have disagreed with him. The prevalent view, based on that of Litten, is briefly (dealing particularly with the cases of calcareous degeneration in the kidneys) that one or more irritants act on certain tubules in the kidney through which these irritants are excreted, and during their passage through the kidney cells,

so injure the tubular epithelium that a degeneration of the cell protoplasm occurs, and that this degenerated cell protoplasm has an affinity for, and fixes as an insoluble compound, the calcium salts brought to it by the lymph and blood. These, it is maintained, are the essentials for the deposit of calcium salts, and it is pointed out especially that a coagulation necrosis of the renal epithelium is a necessary stage in the process.

This view has been generally accepted; and yet it is noteworthy that no one has proved that outside the body there is to be obtained the formation, even temporarily, of compounds of lime and any form of proteid. The present investigation was undertaken, in the first place, to determine with what organic substance present in degenerating tissues it could be shown that lime salts entered into combination. It was in repeating Litten's and von Kossa's experiments and in studying also the changes occurring within celloidin capsules containing various substances, and introduced into the peritoneal cavity, that indications were gained leading to the results about to be detailed. It was first undertaken to produce, experimentally, calcification of the kidney and other tissues and to study the successive changes to be noted in these. The rabbit was most frequently used in these experiments, partly on account of the normally high calcium content of the blood, and partly because these animals stand various operations very well. But in addition many calcified tissues from the human body were studied, the process in connection with arterio-sclerosis especially, and, as will be detailed, not a few experiments of other nature were made during the course of the investigation.

In studying the calcareous degenerations, the tissues were subjected to a series of tests as to the staining qualities and the chemical nature of the material. From all the experimental work on animals and the analyses of human degenerating tissues, one conclusion was always arrived at, namely, that calcareous degeneration is preceded or accompanied by deposits of a soapy material. This soap exists in chronically inflamed tissues in advance of the deposit of lime, or, if the lime is already to be found in the tissues, the soap appears in the peripheral zone

of the calcareous infiltration. Moreover, it would seem that with the first deposit of soap, calcium salts are immediately attracted to it from the body fluids. Such progressive calcareous deposits are marked by the appearance in the periphery of groups of granules, some of which are found to react for both calcium and soap, others for soap only,—that is, some of the soap deposit has been converted into a compound containing calcium; in other words, calcium exists in the recently calcified areas as calcium soap.

That this calcium soap does not continue to exist as such was noted in old calcified fibroids and other tumors. In these it was seen that, except in the advancing border of calcification, the dense calcareous deposits are composed chiefly of calcium carbonate and calcium phosphate with no soap in its substance. It would seem that the fatty acid moiety of the calcium soap had here been replaced by the stronger phosphoric and carbonic acids to form the more stable compounds.

This is, in brief, the trend of the paper.

Before proceeding with the description of the work I wish to extend to Professor Adami my best thanks for the supervision of the entire work and for his unfailing help and the many suggestions which have been of great aid to me during the course of this and other researches.

STAINING METHODS.

Calcium and Calcium Compounds.—As a routine all specimens were stained with hematoxylin and eosin. These stained specimens gave the general character of the section, and, moreover, the presence of calcium salts, when in fair amounts, was indicated by the appearance of fine blue-black dust-like granules, which stained darker than other portions of the tissue and were easily recognized. The hematoxylin will, however, only stain calcified areas when calcium salts are present in very fair quantities; slight amounts are unrecognizable by this stain.

To demonstrate calcium salts in tissues we have two other methods, one of which was of the highest service in my hands.

If tissues containing lime salts be treated for five minutes with a solution of pyrogallic acid (1 to 40 in water), to which one per cent. of sodium hydrate has been added, the lime salts will be stained a dark seal-brown while the remaining tissue appears a light-yellow color. Except in tissues where large quantities of lime salts are present, the stain is unsatisfactory, just as in many cases which show the presence of calcium salts with the following method the pyrogallic acid treatment gave no results. The most satisfactory method we have used is that recommended by von Kossa, namely, the treatment of sections with a 5 per cent. aqueous solution of silver nitrate. This solution picks out the most minute quantities of calcium by depositing an intense black precipitate in its place. The sections were immersed in the silver solution for from 3 to 12 hours, then washed in distilled water, and mounted in the usual way in Canada balsam. As the silver treatment of sections consists of an impregnation and precipitate in the tissues, and not a stain, we obtain a much exaggerated picture of the actual quantity of lime salts present. However, this is of all the more service when minute quantities of earthy salts exist, and the method is not proposed as affording a quantitative measure.

As was pointed out by von Kossa, the blackening produced by the treatment of sections with silver nitrate is due to the production of silver phosphate which blackens in the presence of light; hence it would seem that a precipitation of silver is only obtained in the presence of a phosphate. The calcium deposit in the tissues exists, however, both as a carbonate and a phosphate, principally the latter, and this experiment would only lead us to detect the insoluble calcium phosphate. However, when sections containing calcium carbonate are treated for many hours with silver nitrate a certain portion of the two salts interact so that the calcium-carbonate granules become coated with a deposit of silver carbonate which in sunlight gives off carbon dioxide leaving the black silver oxide, or we can hasten the stage of blackening when some silver carbonate has formed by first thoroughly washing the specimen in distilled water and then treating it with a dilute soluble sulphide.

There is, however, I would point out, a means whereby the calcium existing in any favorable combination in the tissues can be demonstrated by the silver-nitrate method; and this consists in first converting calcium salts into the chromate by means of potassium chromate and then replacing the calcium by the silver, thus obtaining the silver chromate which blackens on exposure to sunlight. Under ordinary conditions the calcium chromate is soluble in water, but when tissues containing calcium are acted on in the cold we find that the calcium chromate does not leave them, and on further treating them with silver nitrate the red silver chromate is seen to become deposited in their sites. The use of silver nitrate in these impregnation tests is we have found facilitated by acidulating it with acetic acid. This same method demonstrates calcium when combined with fatty acids to form soaps.

I would point out that these micro-chemical tests for calcium do not demonstrate the actual calcium salts, but by replacement we demonstrate their presence and location with blackened silver salts.

Virchow and Halva have both analyzed calcareous nodules by chemically demonstrating the presence of both phosphate and carbonate of lime. However, they point out that the phosphate is much in excess; while Chiari found only phosphatic salts in the calcified nodules examined by him.

The method of staining lime salts with silver nitrate gave beautiful pictures in all of the tissues examined. Especially was this true of the sections of experimental calcification of the renal tubules (vide Fig. 2, Plate xxx.). It is true that the metals, mercury, copper, and lead, will give a black precipitate in tissues with silver nitrate, but they can be excluded in these cases when other evidences are given of the identity of the calcium.

However, in the experimental production of calcium deposits in the kidney we have to deal with another factor not often met with in the slowly forming calcareous nodules. In the former we are inducing a sudden change by means of chemical or mechanical reagents, and this has an effect on the general metabolism. Especially is this so in the inoculation of animals with such

poisons as corrosive sublimate, lead acetate, or copper sulphate. Here besides acting directly on the kidney tissue we produce an hæmolysis of the red blood cells and thus occasionally, depending on the strength of the inoculation, we find an iron deposit in the same convoluted tubules, which are the seat of calcareous degeneration. Similar though less amounts of iron can be demonstrated in the liver; but that the presence of iron in the kidney is not the cause of the silver deposit, is to be ascertained from the fact that no calcium reaction results in the liver. The iron in both instances is detected by the Prussian-blue test. Attention has already been called by Gierke to the occasional presence of iron in specimens of pathological calcification.

In the routine examination of aortas we were struck by the frequency of stainable calcium salts found in either the media or intima where no lesion was suspected from the macroscopic or ordinary microscopic examination.

Fats and Fatty Compounds.—For the detection of fats, fatty acids, and soaps, I found that Sudan III gave the most satisfactory results. I have also used Scharlach R., which is a very closely allied stain to Sudan III, but found a difficulty in the employment of it as the stain precipitates very readily in its crystalline form into the tissues. Scharlach R. is the more intense but cannot be used in saturated solutions. Osmic acid impregnation of tissues to demonstrate fats is of more limited use, as we know that only the oleic acid and olein are blackened by it, while stearic and palmitic acids with their respective fats are left untouched. Hence in this work, outside of making comparisons of the quantities of olein or oleic acid and the total quantities of fats and fatty acids, sections were stained with Sudan III; osmic acid was used only very occasionally. The nature of Sudan III and Scharlach R. is such that they are insoluble in water but are soluble in alcohol, chloroform, ether, fats, fatty acids, and soaps. A saturated solution of the stain is readily obtained in 70 per cent. alcohol, and as the fats, fatty acids, and soaps are more ready solvents of it than is 70 per cent. alcohol, fat in sections treated with a saturated solution of Sudan III will pick out the stain while the rest of the tissue remains

untouched. Our sections were usually stained 12-24 hours in the cold. (Vide Fig. 1, Plate xxx.)

Hence in Sudan III we have a stain which will differentiate fats, fatty acids, and soaps from other products in tissues. That soaps are ready solvents for Sudan III is readily demonstrated by making a concentrated watery solution of a soap and then adding the powdered Sudan III. The resultant mixture is a clear, deep red solution which can be filtered and further diluted. Such a stained though highly dilute solution, when evaporated on a slide gives a pinkish-yellow color, which is distinct from the golden-red staining of neutral fat. Fat particles even when emulsified to fine droplets retain this intense golden red appearance. The difference between the Sudan III fat stain and Sudan III soap stain is quite readily recognizable so that the two can be detected in tissues. It is more difficult to distinguish the neutral fats from the fatty acids in stained smears under the microscope, though olein appears distributed over the slide as stained oil globules which are easily picked out. These latter can be further differentiated by treatment with osmic acid.

It is to be noted that fatty acids as such are infrequent in the tissues, except in the case of dead tissues, such as gangrenous areas and pus, or as in the process of splitting up of fats in what are termed fat necroses. It is obvious that living cells do not pick up fatty acids unless, according to Fischler, it be leucocytes, in which case we can at times demonstrate fatty particles in the cytoplasm and show the vacuoles about the particles to be of acid reaction.

Fischler is most emphatic in his assertion that Sudan III is soluble in soap solutions, and the color produced on solution distinguishes this substance from the fat when stained in the same way, or, in other words, that fat and soap granules are to be differentiated in stained tissues. My results wholly confirm these statements. He states, further, that soap, when stained with Sudan III and then treated with 70 per cent. alcohol, immediately loses its color, the dye being taken up by the alcohol. This I failed to confirm wholly.

The statement is true of soap preparations in the test-tube, but, as we shall see later, soaps present in the tissues do not dissolve so readily in weak alcohols; hence they retain the stain after this treatment. As will be pointed out, these intracellular soaps do not exist as a free product, but form combinations with albumin with coincident alterations in reaction.

Calcium soaps are also stained by Sudan III, giving color reactions similar to those of potassium and sodium soaps. Calcium soaps, appearing in the tissues as a new deposit in which there is no excess of free calcium salts, are not distinguishable from other soaps. But when a portion of the calcium is freed and deposited either as carbonate or phosphate, we find, among the Sudan-III-stained soap granules, other, crystalline, bodies, highly refractile and not absorbing the stain. The latter granules, it is found, will precipitate with silver-nitrate solutions. In tissues therefore it is possible to treat the sections first with Sudan III, and later with silver nitrate, demonstrating the presence of soap granules and also showing amongst these free calcium salts not associated with fats.

Some might be sceptical regarding the ability to distinguish the finest fat particles from the granules of soap stained in sections. Such scepticism we can override by another method. If the material be cut by the freezing microtome, the sections quickly dehydrated in 70 per cent. alcohol, then washed with petroleum ether, the tissue may be rid of all the microscopic fat. If now soaps be present, the soap reaction with Sudan III will still persist. I do not wish to say that the ultimate fat is removed from the tissue, but all the stainable fat is taken out, and the petroleum ether has been without action on the soaps. Rosenfeld and Dormeyer have pointed out that tissues which do not show fat microscopically, when washed with ether or petroleum ether, still contain a considerable quantity of fat present within the cells; but the combined fats of this order give no reaction with Sudan III; they may be neglected in this regard. Here it may be noted that even after decalcified tissues have been prepared in the usual way for cutting in paraffin, by dehydrating in absolute alcohol and clearing with xylol, sections will still stain

faintly with Sudan III in the regions of active calcification. I cannot but believe that in such cases we have to deal with soaps rendered less soluble by their combination with albumins (vide p. 659).

CHEMICAL METHODS.

The Determination of Soaps.—In our first experiments the method of Hoppe-Seyler was employed. The tissue under examination was finely ground up and extracted with boiling absolute alcohol. From the filtrate, evaporated to dryness, fats were later removed by ether or petroleum ether, and the residue was demonstrated to contain sodium and potassium soaps, along with small quantities of calcium soap. The residue on treatment with mineral acids yielded stainable fatty acids, all dissolving in water; the sodium and potassium soaps were precipitated with calcium chloride. As calcium soaps are so slightly soluble in alcohol, only small quantities of them were thus obtained and we had to rely on the flame test to demonstrate the calcium present.

The use by this method of hot alcohol for the extraction leaves it possible, perhaps, that a small amount of neutral fat may be converted into soap by means of the alkalis present in the tissues. As I was, however, dealing with calcified tissues, such as fibromata of the uterus, from which all the surrounding superfluous tissue had been removed and which were then washed with distilled water, I can hardly believe that sufficient sodium or potassium remained in the tissue to give rise to the formation of soluble soaps.

To obviate this possibility of error, the method was given up and the fats were extracted without the use of heat. After the material had been finely chopped it was dried over sulphuric acid and then extracted with petroleum ether in many relays, the powdering of the tissue being undertaken between each treatment with fresh petroleum ether. Dormeyer is very emphatic in his assertions that this method of treating tissues with ether, even when carried on for a long time, does not remove the last trace of neutral fats contained within the cell membrane. He

prefers to digest with pepsin and 0.5 per cent. hydrochloric-acid solution, and thus break up all the cell bodies, whereby he claims to obtain 8.5 per cent. more of fat.

As the analyses here undertaken were not of a quantitative but only of a qualitative nature, these minute estimations are of no bearing. However, the gross fats being once removed, the powdered residue was treated with hot acid alcohol to dissolve out the soaps (which had been unaffected by the petroleum ether), and a filtrate obtained. From the filtrate the soaps were precipitated with acid calcium-phosphate solution, filtered off, and washed with ether. In tissues actively undergoing calcification, this residue was obtained in fair quantity—in fact, in greater quantity than in densely calcified tumors in which the amount of the calcium salt was altogether out of proportion to the fatty acid. The alcoholic extract of the residue, after treatment with ether and petroleum ether, yielded small quantities of calcium soap, which we must remember is only very slightly soluble in absolute alcohol.

THE PROCESS OF DEPOSIT OF CALCIUM SALTS IN BONE.

In Normal Bone.—In the above description of the methods employed I have had to refer to the results obtained, and, if only in a general way, I have indicated that, in tissues undergoing calcification, fats, soaps, and insoluble calcium salts are found associated in varying proportions, according to the stage of the process. We will now turn to a more detailed study of the process, and first consider whether under normal conditions—when calcium salts are laid down in bone—there is to be made out this same association; whether, in short, ossification and pathological calcification are processes of the same order.

The phalangeal bone of an infant six days old was examined by sectioning on the freezing microtome and staining with Sudan III. The head of the bone was cartilaginous with a centre of ossification within it. The sections were allowed to stain for eighteen hours, but at the end of this time did not take the Sudan III stain in any part. Both the cancellous and the compact bone were devoid of anything resembling fats, fatty acids, or

soaps. The femur of a foetal guinea-pig was next sectioned and treated in the same way and with like results; that is, we found that in normal growing bone there appears to be no stage in which fatty substances play any part in the deposition of the lime salts, and we are led to believe that this laying down of calcium salts in the bone is a secretory process on the part of the osteoblasts. Again, in sections of decalcified skull bone we found the matrix entirely devoid of microscopical fatty substance. The bone corpuscles, or lacunæ, remained unstained with Sudan III; nevertheless there was some neutral fat lining the Haversian canals and surrounding the contained blood-vessels. Thus we conclude that *the process of calcification in normal growing bone is a distinct process in which the specific cells of the part play the chief rôle in laying down the calcium salts.* There was nothing in the stained tissue which would lead us to believe that there is any connection between fatty substances and the advancing bone-formation at the epiphyseal line.

Pathological Bone Formation.—What is true with reference to normal bone was found to obtain also in new bone developed under pathological conditions. For this purpose we examined a small bony nodule developing in the tissues to the inner side of the lower end of the humerus, secondary to trauma and due apparently, as pointed out by Dr. Archibald, to a local separation of portion of the periosteum. In the second place, we examined a portion of a large nodular, pure osteoma of the left frontal bone, obtained from a man twenty years old, which had grown into the cranial cavity and had produced great atrophy of the frontal lobe. [Royal Victoria Hospital—P. M. Reports, No. 15/05.] In neither of these specimens did Sudan III give any trace of the presence of fat or soap in connection with the more recently growing portions.

THE PROCESS OF DEPOSIT OF CALCAREOUS SALTS UNDER PATHOLOGICAL CONDITIONS.

In the Arterial Wall.—We now pass on to tissues which, under pathological conditions, undergo *calcareous degeneration.* Of

these first and foremost are the arteries. All specimens examined by us, coming from persons over twenty-five years of age, showed more or less change in the aortic wall. The change varies from the faint white streaks which are seen through the intima, to the condition in which calcareous plaques are present, with and without ulceration.

The most remarkable point that strikes one in staining a series of aortas by the methods described, is the frequent presence of recognizable amounts of calcium salts in the media of adults. (Vide Figs. 3, 4 and 5.) In the routine examination of eleven specimens of the aorta, from persons varying in age from twenty-two to forty years, which on macroscopical examination showed no lesion other than fatty plaques in the intima, it was found that calcium salts were present in abundance at one time in the media, at others in the intima. It was not infrequently seen that the media was affected by the calcium increase while the intima remained unchanged or was affected by a slight extension only of the process from the media. We hope to call fuller attention to this change in the media in a subsequent article. So far it would seem to have escaped notice.

The white plaques in the intima have long been noted by pathologists and have passed under a host of names. Gross, as early as 1845, spoke of the condition as "atheromatous" and "steatomatous," where he says the microscope "brings into view a multitude of crystalline plates and fatty granules with albuminous and oily particles." Coplin has collected cases in which the change in the arterial wall was noted as early as the second year of life, but it is not our intention to deal here with the frequency of this affection. What is to be noted is that all vessels with arteriosclerosis have stainable calcium salts in either intima or media to a greater or less extent.

In studying normal aortas, taken from young adults, we found that neither intima nor media was affected by Sudan III, while only a few fat cells were seen in the adventitia. That is, in a normal and healthy state the intima and media of the aorta show no "microscopic" fat, and, further, when such aortas are stained with silver-nitrate solution there is no staining or deposit of silver

in the tissues. Obviously the small amount of calcium salts and fat normally present in the tissues is not detected by our staining methods.

Sections were made from the iliac artery of an adult aged thirty years, which was apparently healthy on gross inspection, with the exception that here and there white areas of the size of a pin-head and not raised above the surface were present in the wall. The specimen was one which had been picked from a number as a sample of an apparently normal large vessel of adult life. When stained with hæmatoxylin, it was seen that the elastic layer did not follow the lumen of the vessel at an equal distance around the artery, but at the sites of the macroscopic fatty areas the intima was thickened towards and bulged into the media, and here the elastica was distorted and at times broken. Away from these isolated changes in the intima, the artery was found to be perfectly healthy. When stained with Sudan III, it was found that these thickenings showed the presence of numerous fatty granules, many of which stained an intense reddish-yellow. This fatty material lay in aggregated clusters of granular particles of larger and smaller size, situated chiefly at the border-zone of the intima, next to the media. While these fatty particles were seen in the nodular portion of the intima, the rest of the arterial wall was free.

In the healthy portion of the arterial wall the elastic lamina did not stain with Sudan III, and followed the lumen of the vessel at an equal distance and in a single strand. As we approached the intimal lesion, the elastic layer split longitudinally into a number of strands, which now, instead of retaining a regular wavy contour, became of unequal undulations and spread apparently into the media. The individual strands of the elastic lamina were swollen and fragmented, showing in places "a snapping" of the fibres. In this area of change in the elastica, the fibres were also seen to take the Sudan III stain, which became more intense and more granular as we approached the thickening of the intima. Immediately below the fatty deposits in the thickened intima, the elastic fibres were found much split up and composed of short, curly, Sudan-III-staining, strands.

When we come to examine closely the particles stained by Sudan III, we find them of different characters. First, we note the larger droplets or granules which are not perfectly round and which are isolated, or else we see several of these fusing into larger masses. The color of these is unmistakably that of neutral fats. Besides these we find many more smaller particles which are distinctly semi-crystalline and of a much lighter color, approaching almost to pink. These pink-staining particles are found in greatest abundance along the border-zone between the intima and media, and often occupying the elastic lamina itself.

It was found that when consecutive sections were stained alternately with Sudan III and silver nitrate, the silver nitrate was precipitated in areas identical with those picked out by Sudan III, though the relative quantities were unequal, there being proportionately more fat present. That is, the calcium deposit picked out by the silver nitrate occupies the nodular portion of the intima and is not seen in the rest of either intima or media. Further, the portion of the thickened intima picked out is that occupied by the pink-staining granules. In the less affected parts the calcium appears as fine dust-like material staining an intense black. Judged by the outline, seen as a silhouette against the lighter background, the individual granules here are crystalline. In places the elastic lamina was picked out as a wavy line composed of aggregated granules of calcium.

In this specimen we have the early change in the aorta, a condition which we would not yet speak of as arteriosclerosis. The white pin-head patches on the intima are seen to represent areas with fatty substance of two kinds: one an intensely yellowish-red staining material, which is fat; and lighter particles, which from their reaction with Sudan III can only be soaps; and, further, the thickenings in the intima are shown to contain an increase in the calcium content, and this increase to occur in conjunction with the light, soapy staining material. In other words, the calcium increases side by side with the soap; and the arrangement described suggests strongly that we are dealing with a deposit of calcium soap.

In another instance there was a generalized thickening of the

arteries, though only at the bifurcation of the aorta was any macroscopical calcareous degeneration to be seen. The aorta showed numerous white plaques raised above the surface, and one of these from the abdominal aorta was sectioned. Stained with Sudan III both media and intima showed numerous aggregations of stained particles; the staining in the intima was confined chiefly to the atheromatous thickening in which considerable fat-staining masses occurred, while outside the nodule the granules were collected mainly round the elastic lamina. As in the previous case the granules could be divided into two classes: the larger masses which fused with other contiguous particles, readily recognized as fat deposits; and the smaller, lighter-staining granules which appeared semi-crystalline in shape. In the intima the larger fat masses were in excess of the finer granulations, while in the media the reverse condition existed; that is, the light-staining granules were much in excess of the fat, in fact only a little fat was to be seen on the boundary between the media and intima. In the rest of the media there were clusters of fine sand-like particles lying between the elastic layers and forming rows, more or less wedge-shaped, which occupied the position of the muscular fibres. In the media the stained granules were intermingled with numerous unstained refractile bodies, which disappeared from the specimens when treated with acid solutions. On treating the sections with silver nitrate it was found that the blackened deposit was present in the media in the regions where the Sudan III demonstrated the light-staining soapy particles. Similar to the Sudan III granules, the silver-nitrate deposit was aggregated into clusters lying parallel to and between the elastic fibres. The silver-stained particles in these clusters were much in excess of the refractile bodies seen in the Sudan III specimens; they tallied in number with those seen in unstained sections. The distribution of the silver-stained calcium salts was fairly general through the media, the greatest amount being towards the internal surface. (Vide Figs. 3 and 4.)

In the intima, however, there was less calcium to be seen, that present being confined to the atheromatous areas where the deposit seemed to be invading from the media. Beyond the

atheromatous plaques, there was no lime salt to be seen in the intima excepting a fine, dust-like infiltration along the boundary zone. The whole condition in the arterial wall we look on as consisting of two distinct processes: the change in the media of a chronic nature and of longer standing than the intimal change, the degenerative process with fatty change having been progressive; and the atheromatous condition in the intima of more recent date, owing to which time had not been given for such extensive deposits of calcium salt.

We now undertook to remove all the neutral fats and fatty acids from the sections of the aortas above described by treating them with ether and petroleum ether. They were first washed in 70 per cent. alcohol before subjecting them to the ether. After this treatment the only material remaining which could stain with the Sudan III would be a soap. On examining the sections stained with Sudan III, it was found that all the bright-red staining neutral material had been removed and all that remained to take the stain were small crystals of a light salmon-red color. These crystals lie in clusters of 50 or more, the long axis of the clusters being parallel to the direction of the fibres. The clusters are to be found mostly in the intima at the atheromatous area and in this tissue lie some little way inwards from the endothelium. In the deeper parts of the intima the clusters become more broken up, and after staining with silver nitrate are seen to be mingled with calcium deposits, which are absent in the more superficial parts of the intima. Hence the Sudan-III-staining particles which remained near the boundary zone between the media and intima after treatment with petroleum ether are combined with calcium salts, while those, apparently of more recent origin, lying near the endothelial surface contain no calcium salt. When we examine the aorta beyond the atheromatous patch, we lose any evidence of soap in the intima except in the deeper portions near the media, and only as the atheromatous area is approached do the layers between the fibrous strands become widened and the spaces become the site of deposit of soap granules. In the media, where, as we have described, the calcium salts are much more marked, similar clusters of granules

to those noted in the intima are found, with, however, the difference that in the media the clusters are compounds mainly of silver-nitrate salts—much calcium with a little soap,—while in the intima the proportion is exactly reversed.

To resume, it may be stated that by employing suitable methods it is found that calcareous deposits occur in the arterial walls, more particularly in the wall of the aorta, at an earlier age than is usually suspected.

These deposits vary as regards the region first affected. This variation may be in regard (i) to the internal elastic lamina, (ii) to the media, more particularly its muscle fibres, and (iii) to the intima in areas of nodose sclerosis.

Judging from the treatment of sections for the demonstration of fats and calcareous salts, the stages in this process are (i) degeneration of the affected region with deposit of fatty globules, (ii) formation of soaps, mainly with calcium, (iii) deposit of calcium salt devoid of a fatty moiety.

The results obtained in connection with calcified deposits in the arterial walls were confirmed and extended by an examination of other conditions of calcareous infiltration.

Calcareous Infiltration of Thickened Pleura.—The cases examined were evidently of some duration. There were thickened fibroid adhesions between the visceral and the parietal pleuræ, and in the centre of the fibroid layers dense calcified plaques occurred. The plaques were found to form rather irregular laminæ with somewhat irregular surfaces. By examining the material after decalcification, the section through what was a densely decalcified plaque was found to be stained a diffuse pale-yellow with Sudan III, no individual granules being present; but in the immediate neighborhood of the plaque was an aggregation of masses stained deeply with Sudan III. Therefore at the periphery where the process of calcification was still continuing there was definite evidence of the presence of fats.

In studying the decalcified sections it was noticed, as frequently observed by others, that the decalcified areas stained more deeply with hæmatoxylin than does normal tissue. Very

few nuclei were recognized. The matrix left behind after removing the calcium salts was of a hyaline nature. The existence of this matrix led Litten and others to the theory that the calcium salts are deposited as calcium albuminates. That the fat or soapy substances, still exist in connection with the matrix is indicated by the light-yellow color given with Sudan III; and remembering that, as we have noted, soap solutions and albumins form insoluble compounds with which calcium can combine, it would seem more probable that we are again dealing in this instance with the resultant albuminous material deposited after the calcium has separated from the combination to unite with phosphoric and carbonic acids, and possibly to some extent also after a part of the soap has been split off to enter into more soluble combinations.

Calcified Fibromata.—A similar condition is found in calcified fibroma, in which the most extensive infiltration is not in the centre of the fibroid mass but in the outermost layer, whence it is proceeding towards the centre. In each of the specimens examined, three zones of change were well marked. In the outermost area (which varied in thickness), when the sections are stained with both Sudan III and silver-nitrate solution there is seen a dense black mass, the small amount of soap present in this zone being wholly obliterated by the black-stained lime salts. At the margin of this zone and farther inwards clusters of granules are to be seen, some of which stain with Sudan III, others with silver nitrate. At times an individual cluster, black at one end, yellow at the other, can be made out. In this area, between the groups of granules can still be seen the wavy fibres of connective tissue. This constitutes the second zone. The third zone, farther inwards, exhibits granules staining only with Sudan III. Thus there is well-marked fatty change, the presence of calcium soap, and finally the presence of calcium salts showing no soapy constituent.

That calcifying fibroid tumors should show a deposit of lime salts at the periphery is a phenomenon of the same nature as has been observed in connection with lithopodia and with kidneys undergoing calcification after complete cutting off of their blood

supply. The fibroid tumor that undergoes calcification is one that is undergoing necrosis through defective blood supply, and the calcareous salts deposited are not those already present in the tissue itself but those precipitated from the lymph which diffuses into the dead tissue from without. It follows that the diffusible salts are taken up by the first soapy substances with which they come into contact in this process of diffusion, and only when those in the peripheral zone become calcified do the soapy and fatty matters situated more deeply have the opportunity to form calcium compounds.

Calcareous Infiltration of Ovarian and Broad Ligament Cysts.—Cysts of this nature, in which a thin shell of lime salts is laid down in the fibrous tissues of the walls, are occasionally encountered. The process is usually confined to a layer bordering on the cyst cavity beneath the lining membrane of the cyst in the surrounding fibrous tissue. In these we have found the usual evidence of fatty change, of soap formation in the immediate neighborhood of the areas, and of deposition of lime salts.

Calcareous Tubercular Nodules.—The nodules can be decalcified, cut by the freezing microtome, and the sections treated with Sudan III or Scharlach R. It has been usual of late years to speak of tubercular caseation as a process of protoplasmic degeneration in which there is very little associated fatty degeneration. It is interesting therefore to note that by employing the methods described by us it can be demonstrated that the caseous areas which have undergone calcification contain a very considerable amount of fatty matter, so much indeed that the yellow staining areas of the tubercular nodule stand out very prominently from the surrounding tissue. Treatment of such sections with ether removes some of the material stainable with Sudan III, but by no means all. As might be expected from what has already been said, differences are to be made out between old and more recent calcified tubercular areas. In dense, old, calcified glands and nodules there is less of the fatty staining material left, and it is only here and there, where the soap moiety has not been wholly removed, that the deposition of the lime salts gives any results with Sudan III, while in softer and less

dense nodules larger and smaller granules staining with Sudan III are abundant. It is noticeable that very little fat is to be made out in the enveloping fibrous tissue. The question naturally arises, Where does this fat come from? Examination of lung tissue in cases of active tuberculosis showed that the fat and its products are already present in tubercles showing the early stages of central necrosis which as yet scarce show caseation. In caseous tubercles this fatty matter is still more evident, and in these also calcareous salts in the form of fine granules are to be detected. It was only in those tubercles in which the fatty substances were aggregated into larger granules that lime salts could be demonstrated. In the earlier non-caseous areas there was no reaction of the calcium; and it is to be noted that there were more Sudan-III-staining granules in the centre of the caseous areas than at the periphery. This series of events indicates the following stages, viz: necrosis, fatty degeneration, formation of calcium soaps, and deposition of calcium salts having no soapy moiety.

Pancreatic Fat Necrosis.—It has been pointed out by various observers that in pancreatic lesions followed by fat necrosis the neutral fats within the fat cells undergo decomposition, and as a result fatty acid crystals are left behind. These fatty crystals after a time unite with the lime salts of the tissue fluids to form insoluble calcium soaps. We found that in the experimental production of fat necrosis the fatty acid crystals could be stained readily enough, but unless the animals experimented upon remained alive for more than a week the lime salts were not to be demonstrated microscopically in the lesions. In other words, the combination of the fatty acids with calcium to form calcium soaps is not an immediate but rather a slow process. A definite period of time seems to be necessary before the calcium soaps accumulate so as to be present in stainable quantities. We examined one specimen of hæmorrhagic pancreatitis in man and found that fatty acid crystals had been deposited in the peripheral zone of the necrotic area. In this zone calcium salts were seen in small amounts distributed as a fine dust. The specimen, however, was an accidental find in a recent autopsy,

two small foci of necrosis in the tail of the pancreas, each scarce one centimetre across, being present, and there was no clinical indication of the length of time that the condition had existed. The earlier observations upon the presence of lime soaps in connection with pancreatic fat necrosis will be referred to later.

The preceding section of this paper may be reviewed as follows:

(1) All calcareous infiltrations are preceded by fatty changes and in them substances are found consisting of neutral fats, fatty acids, and soaps, which stain with Sudan III.

(2) By employing appropriate methods, the microscopic fat and fatty acids can be removed, the soaps remaining behind, the last being detected by reason of their differential staining with Sudan III.

(3) The granules in certain regions which give the soap reaction with Sudan III give also the calcium reaction with silver nitrate.

(4) As the process of calcification advances, many of the masses deposited in the part no longer stain with Sudan III, but only react for calcium salts.

In areas undergoing calcification three zones are to be made out: (1) The oldest zone of complete calcification, exhibiting dense calcareous deposit lying in a hyaline matrix; in this zone little soap or fatty substance is to be demonstrated. (2) The intermediate zone, in which granules of lime salts and soap granules are in close apposition; some of these granules give both the calcium and the soap reaction. (3) The most recent zone, which gives the reaction for fat, fatty acids, and soaps and fails to reveal calcium salts in sufficient quantities to be recognized under the microscope.

ON THE PRESENCE OF SOAPS IN THE ORGANISM UNDER PHYSIOLOGICAL CONDITIONS.

Since the observations above detailed indicate that soaps play a most important part in the process of pathological calcareous change, it appears desirable to compare with them observations

made upon the part played by the soaps in the physiology of the animal economy.

In 1857 Marcet stated that during fat digestion the fats are converted into soaps in the intestinal canal and these soaps are absorbed by the intestinal mucosa. The absence of any amount of soaps in the blood comparable with the amount of fat absorbed led others to conclude that the soaps as such did not pass beyond the endothelial cells, and to suppose that a glycerin molecule is furnished by these cells, the reconstructed fat being passed into the lacteals. Heidenhain pointed out that some at least of the fat is taken up by the leucocytes on the surface of the intestine and is carried by them directly into the lacteals and blood-vessels. More recently, Ramond has denied the validity of Heidenhain's observations, and has concluded that the greatest part of the fat absorbed enters the portal system and not the lacteals, and enters in the form of soap and not of fat. From this statement we infer that Ramond regards the soap as becoming transformed into fat during its passage from the endothelial cells to the portal capillaries. He finds also that the liver tissue contains a ferment capable of splitting fats into their respective fatty acids. Beneke and later Hoppe-Seyler have pointed out that the blood normally contains soap in solution. Beneke makes an interesting suggestion as to the manner of recovery from pulmonary fat embolism. He points out that possibly a solution of the fat of the emboli is brought about through the action of the lipase present in the blood, which converts the fat into a soluble soap. He also states that fat inoculated subcutaneously disappears so rapidly that the only satisfactory explanation is one based on its conversion into soluble soap.

Still more recently Schultz, who studied fats as a whole in the blood, found that no less than 28 per cent. of the total fatty acid obtainable is present in combination as soap. During starvation, when the fat deposits are used up, he found an increase of the fat in the blood and concluded that the fat is transported from its normal deposits by way of the blood. The fat in the tissue cells he holds becomes transformed into soap and thus passes in solution into the blood.

As to the manner of conversion of the fats into the fatty acid, in which form combination occurs with alkalies to produce soaps, we have the observations of Hanriot. This observer found that blood serum, pancreatic juice, and liver juice contain a ferment capable of converting the neutral fats into their respective fatty acids. This ferment was not present in muscle tissue, testicle, or thyroid gland. The substance isolated by Hanriot seemed by its action to be a true ferment, and was present in all the animals examined. He made a distinction between pancreatic lipase and serum lipase, claiming that they are different substances chemically. Cohnstein and Michaels, who also found a lipolytic ferment in the blood, differ from the former observer in believing that it is present in the red blood cells rather than in the serum.

There can be little doubt that the leucocytes and endothelial cells play parts in the synthesis of fat. It is well known that leucocytes are fat-carriers and that they transport fat from the intestinal canal during fat digestion. The quantity of fat transported must be very small indeed, but Arnold has found that subcutaneous injection of soap solutions into the backs of frogs attracts leucocytes which become filled with fat, and he also noted that eosinophilic leucocytes are especially active in this fat synthesis. Fischler has shown by similar experiments that subcutaneous injections of soap are followed by deposit of fat in the organs, and that when a soap solution is passed through the renal vessels the fat particles can be seen between the endothelial cells and in the kidney tissue. Hence it would seem that endothelial cells take part in the conversion of soap into fat. This view is also supported by the statements of Ramond, namely, that the fat from the intestine passes through the intestinal mucosa, as through a dialyzing membrane, in the form of soap and is reconstructed on entering the portal system.

Munk studied the toxicity of soap and pointed out that soap solutions injected into the portal vascular system are only one-sixth as toxic as when inoculated into the peripheral vessels. From this it would seem that the portal endothelia or the liver parenchyma are especially active in converting the soaps into neutral fats.

THE PRESENCE OF SOAPS IN THE ORGANISM UNDER PATHOLOGICAL CONDITIONS.

It is not a little remarkable that very few observations upon the part played by soaps in pathological conditions have appeared in the literature. We have encountered isolated notes only upon the subject, and do not know a single research, properly so-called, in which their rôle has been fully worked out. Many years ago Virchow called attention to the presence of calcium salts in degenerating lipomata, and very correctly concluded that the fats present in these tumors combine with the calcium salts to form soaps. Kyber in 1880 referred incidentally to the part which both alkalies and fatty acids must play in the process of calcification. Somewhat later Jaeckle and others enunciated the theory that the formation of soaps is one step in the process of calcification of fatty tumors. But this theory has to our knowledge never been tested experimentally and would seem to have become obsolete. In 1890 Langerhans pointed out that in fat necrosis following pancreatic lesions crystals of fatty acids appear, and in many cases unite with calcium of the blood to form an insoluble calcium stearate which occurs at the site of the lesion. It does not appear that Langerhans ever continued these observations or expanded his findings so to apply them to calcification in general. Quite recently Fischler has published a preliminary note upon certain methods for detecting the presence of fats and soaps in the tissues; and though he does not state it definitely, he nevertheless seems to imply that his observations point to an important part played by soaps in more than one process.¹

As indicated in the preceding pages, our observations all point to the fact that the formation of soaps is a necessary step in the development of pathological calcification. We may at this

¹ The number, of the *Centralblatt* containing Fischler's article reached America, I may add, after I had forwarded to Philadelphia the abstract of my preliminary communication upon this subject, which was read at the meeting of the Association of American Physiologists in December, 1904. A study of Fischler's paper will show that he has reached his conclusions by the employment of methods differing from those given here.

point present our further studies on the chemistry of this process.

The analysis of aortas which were in the process of calcification yielded calcium soaps in small quantities together with sodium and potassium soaps, though chemical extraction did not give as clear an idea of the process as was obtained by treating sections with petroleum ether and later with Sudan III. Sections so treated seemed to indicate the presence of a fair amount of soaps. By analysis the quantities obtained were very small. Indeed the Sudan III stain after treatment seemed to show clearly that substances which *in vitro* are soluble are relatively insoluble when present in the tissues. For instance, sodium and potassium soaps are, as we know from every-day experience, easily soluble substances, while, on the contrary, calcium soaps are relatively insoluble. We were prepared, therefore, to find that the tissues contain relatively small amounts of the former, and large amounts of the latter, supposing that when formed locally sodium and potassium soaps would diffuse into the blood and lymph and so escape to a very large extent; but to our surprise the sodium and potassium soaps were not nearly so soluble in tissues as they are *in vitro*—in fact they are relatively insoluble in the former location.

ON THE EXISTENCE OF A COMPOUND OF SOAPS WITH ALBUMEN.

What would seem clearly to be the explanation of this difference was discovered in conducting observations in the test tube. Doubtless the reaction which we are about to describe has been noted before, but, if so, it has failed to come to our attention. If to a weak solution of a pure soap—sodium stearate for example, though the same is true of the oleate and the palmitate—there be added dilute egg albumen, a white rather flocculent precipitate forms. The reaction is not immediate: at the temperature of the room it requires half an hour or longer before it is complete; the precipitate is a combination of soap and albumen. The compound is slightly soluble in water and less soluble in alcohol. Under the microscope the precipitate appears granular, and when the particles are treated with Sudan III they assume the

identical pinkish-yellow color of the soap granules observed in tissues undergoing calcification.¹

It is worth noting that the precipitate becomes denser and more pronounced when carbon dioxide is passed through the solution. Acid calcium phosphate may be added to a solution of egg albumen without precipitation; but if the salt be added to a combination of weak soap solution and egg albumen, made as above, precipitation takes place rapidly and yields a dense sediment.

The Chemical Isolation of Soaps from Pus.—We have already stated that we have been able to isolate *soaps* from more than one order of calcified tissue. We were able to isolate a material soluble in absolute alcohol, insoluble in petroleum ether, and precipitable on the addition of acid calcium phosphate from a number of calcified tuberculous glands of the mesentery. On the addition of a mineral acid the fatty acid was isolated from this combination and gave the characteristic stain with Sudan III.

Particular attention is called to the fact that similar soaps may be present in pus. The cases from which the pus was examined were, it is true, of chronic nature; and no opportunity has yet come to us for the study of large quantities of pus of more acute origin. With the pus of two tubercular abscesses (one an empyema, the other a case of Pott's disease), each yielding fifty cubic centimetres, tests were made. Each quantity was extracted with warm absolute alcohol for seventy-two hours, after which the mixture was filtered and the filtrate evaporated to complete dryness. The residue consisted of fat drops mixed with a white granular substance. The residue was collected and washed with ether and petroleum ether for four days, after which no fat or fatty acid could be demonstrated in the filtrate. It was now digested with warm distilled water, and on the addition of

¹ Mr. O. R. Mabie, Phm.B., has undertaken a study of this soap-albumen compound. His studies are at the present incomplete, but he has found that solutions of albumen when mixed with known strengths of chemically pure soap solutions absorb a certain quantity of the soap. He has used albumen from different sources, and with all he has obtained similar results, although different degrees of absorption have resulted.

calcium chloride to a quantity of the watery extract a white precipitate, stainable after evaporation on a watch glass with Sudan III, was obtained. In the unstained condition the precipitate appeared as a white fine powder. Fatty acids were obtained from the residue by adding acid, and the addition of hydrochloric acid to the aqueous solution caused an opalescence in the test tube. Sodium and potassium were detected in the residue by the flame, but no calcium was found. Fatty acids could also be detected and saponified from the extract in the petroleum ether.

ON THE EXPERIMENTAL PRODUCTION OF CALCIFICATION.

In Capsules Containing Fats and Fatty Acids.—Early in the course of this research experiments were undertaken to determine the organic compounds with which calcium salts may unite. Various products obtained as pure soaps were placed in celloidin capsules prepared by the method recommended by McCrae and introduced into the peritoneal cavity of rabbits where they were left for several days. The results of these experiments led to the study of the rôle of fatty acids and soaps in the process of calcification.

Von Kossa places the normal calcium strength of the blood of rabbits as varying from 1.06 to 2.01 per cent., which may be taken as about the limits of range. In our experiments, two celloidin capsules were prepared containing one gramme of sodium stearate and one gramme of sodium palmitate respectively. These were inserted into the peritoneal cavity of a rabbit and allowed to remain there eleven days. On removal, the contents of the capsule were more or less caked and contained some serum. The contents were treated with hydrochloric acid and precipitated with ammonium oxalate, and the solution then rendered alkaline with ammonia. The precipitate was collected and the calcium determined in the ash obtained on ignition. The sodium stearate was found to have combined with the greater quantity of calcium, there being 4 per cent. of the alkaline earth present. The capsule containing the sodium palmitate was found to contain 3.8 per cent. of calcium. Hence it was found that even in the short space of eleven days a definite

conversion of the sodium and potassium soaps into those containing calcium had taken place. Other experiments were carried out in which stearic and palmitic acids were substituted for their salts. The results were similar to the first, there being a decided increase in the calcium strength over that present in the serum of the animal.

The Experimental Production of Calcareous Infiltration in the Kidney.—All cells use fat in some form in carrying out their normal physiological function, and, as has already been pointed out, a great deal of fat is brought to them as soap. When the cell is injured, the soap it would seem is not metamorphosed but remains fixed in the cell in combination with the cytoplasm. This form of union is indicated by experiments on the kidney epithelium in animals, for the main conclusions to be drawn from Litten's experiments are that the tissue cells undergoing calcification are in a degenerating state and have already lost, or are in process of losing, their vitality. He further pointed out that calcification proceeds more rapidly if along with the dead and dying cells there should remain a good circulation to supply the calcium and other materials which are eventually deposited as a permanent precipitate. As Litten examined his sections only with hæmatoxylin staining he found no evidence of fatty products laid down in the areas of his "coagulation necrosis."

The animal experiments reported by Litten were the first dealing with the question of the calcification of the renal tubules. They demonstrated that, if the renal vessels were ligated for two or more hours and then loosened, after eight or ten days crystals of lime salts were to be found in the cells of the convoluted tubules chiefly. The better the blood supply to the organ after ligation, the more quickly did the calcification proceed. The explanation offered was as follows: arrest of the blood to the kidney having caused "coagulation necrosis" of the tubular epithelium, the later advent of the calcium-containing tissue fluids brought about precipitation in the tubules of "calcium albuminate." Although this theory of Litten has been current for twenty years, it is noteworthy that the production of calcium albuminate had not been demonstrated *in vitro*.

We must, however, conclude that in the cells which are injured there is a change in the protoplasm, and of this there is adequate evidence found in the fact that the nuclei stain poorly and the cell substance appears swollen and homogeneous. The alteration of the nucleus is perhaps the chief indication that the cell has suffered grave metabolic changes. There is no evidence at this point of the process that the cell substance is diminished in amount—indeed, on the contrary, the actual amount of cell material appears increased. This is in keeping with the recent observations which point to the conclusion that fat appearing in the cell is not the product of cytoplasmic decomposition, but is absorbed from without. The view we wish to emphasize is that in the degenerating cells absorption of material from without still continues whether this material be albuminous or fatty. But owing to nuclear disturbance, or more broadly to lowered metabolic activity, the substances so absorbed cannot be used up; they no longer become combined into the cell cytoplasm or bioplasm proper, and remaining in an uncombined state may now become subject to various pathological changes.

Neuberger has shown that a number of chemical irritants produce the same effect on the kidney as ligating the renal vessels, and that they lead to calcification of the same sets of tubules. We repeated Neuberger's experiments, using the salts of the heavier metals, mercury, copper, lead, etc., with the result of producing calcareous degeneration in the convoluted tubules of the cortex of the kidney. The rabbits were inoculated subcutaneously with solutions of the soluble salts of these metals and then killed after ten days. A small portion of the kidney was removed for microscopical purposes. Frozen sections were stained with Sudan III and silver nitrate and both gave satisfactory pictures. In consecutive sections it could be shown that the degenerated tubules which reacted for the fatty substances with Sudan III also reacted with silver nitrate; and, moreover, that when a section was treated first with Sudan III and after staining was subjected to silver-nitrate solution, all the Sudan-III-staining tubules were covered over by the intense black stain of the silver salt. That is, the identical tubules which

were shown to contain a fatty-staining substance also contained calcium salt (vide Figs. 1 and 2, Plate xxx). The calcium could be wholly removed from the tissue by the use of dilute acids without affecting its fatty-staining qualities. If frozen sections were treated first with seventy per cent. alcohol and later with petroleum ether, it was still possible to find the tubules picked out with Sudan III and silver nitrate. Finally it has been possible to extract from the cortex of the kidney, potassium, sodium, and calcium soaps. These results indicate that the formation of a calcium soap is one stage in the process of calcification in the kidney; that, degeneration and death of the cells being effected, the cells are not able to utilize the potassium and sodium soap brought by the blood, and hence these are precipitated in combination with albumens, and later the calcium from the tissue fluid and blood is fixed in an insoluble state by this compound. While we agree with Litten, Neuberger, von Kossa, and others, that necrobiosis, improperly referred to by them as a "coagulation necrosis," occurs, we look upon this as only one stage in the whole process of calcification.

Von Werra believes that such degenerated cells of the kidney may return to a normal condition. We can hardly accept this view, as calcareous degeneration of cells is the last stage in a pathological process in which the cells and nuclei are disintegrated. Hence it is improbable that they can regain their vitality.

Tartarini-Galleani claims to have studied the kidneys of rabbits after injecting bichloride of mercury into the substance of the organ. He states that the lesion produced is a tissue destruction followed by fibrinous coagulation, which after the ninth day becomes impregnated with calcium salts. The calcification in the kidney remains circumscribed to the area of the organ directly affected by the injection, and he holds that it is neither preceded nor accompanied by the phenomenon of fatty change. We have repeated his experiments in two rabbits and in each the result was different from that described by him.

Sections of the inoculated kidney treated with Sudan III showed the cells of the convoluted tubules to be swollen and

filled with fatty granules. The tubules were well outlined from the surrounding tissue by reason of the stained fat in the cell contents. The glomeruli and the interstitial tissue appeared unchanged, while the arterioles of the cortex showed Sudan-III-stained granules in their walls. These granules were distributed more loosely than was seen in the epithelium. The affected convoluted tubules presented collections of irregular granules staining not so intensely as neutral fat, but rather a brownish-yellow. The tubules in the pyramids showed a striking difference. While they were less affected than in the cortex the condition of the fat in them differed from that of the convoluted tubules. The individual cells showed no change in size or shape, and the nuclei retained their staining power. However, the Sudan III picked out numerous fine globules situated towards the inner margins of the cells—that is, towards the lumen and away from the nucleus. In some cases it appeared that the fatty globules were in the process of being extruded into the lumen. The Sudan III granulation noted in the straight tubules was of the character of neutral fat. No calcium deposit could be demonstrated in the straight collecting tubules, while in the cortex the convoluted tubules which showed the Sudan III granulations reacted to silver nitrate in the manner of calcium salts.

Résumé.—Our studies upon experimental calcification in the kidney confirm and extend the observations made upon calcareous changes in human tissues. If the tubules become the seat of the calcareous deposit it is possible to recognize the following stages, viz:

(1) A stage of cell degeneration characterized by swelling of the cell substance and diminution of the nuclear chromatin, the cells becoming swollen and homogeneous;

(2) A later stage in which fat appears in the cells, apparently having arisen by absorption.

(3) A final stage in which calcareous salts appear in the cells accompanied by soaps. An intimate relation between cytoplasmic degeneration and the formation of insoluble soaps would seem to exist. Judging from observations made in test tubes,

the relatively insoluble soaps are compounds between soap and certain albuminous matters liberated from the cytoplasm. The calcium from the tissue fluids and the blood becomes fixed by the albumen-soap compound.

Experimental Calcification of Muscle Fibres.—That muscle fibres may become the seat of calcareous deposits is well known. We inoculated a weak solution of bichloride of mercury into the quadriceps muscle of a rabbit, killing the animal at the end of eight days. On examination the muscle at the site of the inoculation showed extensive degeneration and softening. Individual fibres were swollen and in some places the sarcolemma was broken. Other swollen fibres showed band-like constriction. Still other muscle fibres were completely broken down and invaded by numerous polymorphonuclear leucocytes.

The degenerated muscle fibres proved an interesting study. A fine granulation not found in healthy fibres, and staining in Sudan III, occurred in their substance, besides which leucocytes loaded with fat globules invaded the degenerating fibres and lay in the interstitial tissue. The fatty-staining material within the leucocytes appeared in the form of large and small granules, but these did not coalesce to form large fat globules as is seen in adipose tissue.

The question arises as to whether the leucocytes are bringing the fat to the injured tissue or taking it away. We are inclined to the latter view, for among the degenerated fibres were some in which the sarcolemma was intact and which were free from leucocytic infiltration, but which nevertheless contained granules staining with Sudan III. Moreover, the leucocytes at the margin of the injured tissue contained no fatty-staining material.

We think it probable that this process is not unlike that described in the kidney, namely, a state of lowered vitality of the individual cells through which, being no longer able to deal with the fatty material brought to them in solution, they form with it a relatively insoluble compound in the cytoplasm of the degenerating cells.

Experimental Fat Necrosis.—In order to investigate further the changes which fatty products undergo in the body, we pro-

duced fat necrosis in a rabbit by the intraperitoneal inoculation of fresh extract of pancreas. The injection was repeated on the second day and the animal killed on the fifth day. At the post-mortem a few tags of fat necrosis about the size of a pea were found scattered in the mesentery and the posterior abdominal wall. Frozen sections of these showed that the fat did not exist in the large distended fat cells but that the globules were replaced by numerous fine crystalline particles staining with Sudan III. On treating the sections with silver nitrate, the sub-peritoneal margins of the fat necrosis showed the presence of a slight amount of calcium salts appearing like a fine dust-like deposit. All the areas of fat necrosis did not show the presence of calcium with silver nitrate

As already stated, Langerhans showed in 1890 that calcium salts appear in the areas of fat necrosis. He describes the process as one in which the fat is converted by ferment action into fatty acid. The calcium salts of the blood then become attracted to the fatty-acid moiety and form a calcium soap.

ON THE SOURCE OF THE FAT IN THE DEGENERATED CELLS.

Before concluding, it is, we think, essential to indicate our views regarding the source of the fat present thus in all cells that undergo calcification and essential for the due development of the process. To discuss the matter adequately would demand that the numerous recent studies upon fatty degeneration be passed in review: such full discussion would carry us too far afield. It must suffice that we call to mind the reasonable deductions from the work of recent observers which appear to bear immediately upon our present problem.

We may, I think, in the first place safely accept that fats are absorbed by the cell from the surrounding medium in the form of diffusible soaps. Taken up by the cell these soaps may either be reconverted into neutral fats and stored as such in the cytoplasm, or may undergo assimilation proper, becoming part and parcel of the cell substance. That such assimilation does occur with associated loss of individuality on the part of the fats is,

we hold, demonstrated by sundry recent observations. Thus Rosenfeld and others have shown that kidneys presenting no fat recognizable by microchemical means will, nevertheless, yield upon careful analysis as much as fifteen per cent. of fats; and Doremeyer, that mere extraction by ether will not liberate the greater part of tissue fats: the cells have to be digested with pepsin before all can be isolated; a clear indication that the fats have previously been in combination with proteid substances in the cells.

If, then, these possibilities be accepted, it follows that the appearance of fats and soaps in a degenerating cell may be from at least two sources, namely:

(1) The absorption of soaps from the circulating lymph and blood, the cell still retaining the power of splitting off the fat, but being unable to utilize this in metabolism, so that it becomes stored up in a non-diffusible form.

(2) Disintegration of the cell substances so that the fats become liberated from their combination with the cytoplasm and appear in the form of discrete particles, or globules, of neutral fat, which now reveal their presence by reacting with Sudan III.

A third possibility has to be kept in mind, namely, that soaps may be absorbed from the blood and lymph by cells with vitality so depressed that no conversion takes place of the absorbed soap into neutral fat, but combining with the disintegrated albuminous molecules this forms a relatively insoluble soap-albumen compound.

Into the controversy regarding the formation of fat by the decomposition of the albuminous molecule I will not enter, save by suggesting that the opposing views may possibly be harmonized through the demonstration here afforded, that albumens form a combination with soaps. The increase in fats determined in tissue autolysis by Waldvogel, Hildesheim and Leathes, Landsteiner and Mucha, and others is, it may be, to be explained, not as due to the appearance of a decomposition product of the albumen molecule proper, but as brought about by dissociation of this albumen-soap compound.

The time is not ripe for, neither do our personal observations

up to the present permit, any discussion of the part played by cell ferments in any of these processes.

As to which of the above alternatives—absorption or dissociation—is the more frequently in action as a precursor of calcification, we would point out that when the cell becomes distended with fatty globules (and this is common in atheromatous areas) we must conclude that absorption from without is in the main responsible for the condition.

CONCLUSIONS.

It will be seen from the above that we have studied the conditions associated with the deposit of calcareous salts: (1) in connection with normal and pathological ossification, and (2) in pathological calcification as exhibited in (a) atheroma of the vessels; (b) calcification of caseating tubercular lesions; (c) calcification of inflammatory new growth, and (d) degenerating tumors; and we have induced experimentally deposits of calcareous salts in the lower animals: (a) within celloidin capsules containing fats and soaps; (b) in the kidney, and (c) in connection with fat necrosis.

I. We have found that bone formation and pathological calcareous infiltration are wholly distinct processes. In the former there is no evidence of associated fatty change, and the cells associated with the process of deposition of calcium are functionally active. In the latter there is an antecedent fatty change in the affected areas, and the cells involved present constant evidences of degeneration. The view that would seem to account best for the changes observed in the latter case is that with lowered vitality the cells are unable to utilize the products brought to them by the blood, or which they continue to absorb, so that the normal series of decompositions associated with their metabolism fails to take place and hence they interact among themselves in the cytoplasm with the result that insoluble compounds replace soluble ones.

II. Besides the fact that calcification is always preceded by fatty change within the cells, another fact should be emphasized,

namely: that combination of the fats present with calcium salts to form calcium soaps tends to occur. The stages immediately preceding these are difficult to follow with anything approaching certainty, perhaps because the earlier stages vary under different conditions. In fat necrosis, for instance, the cells affected are normally storehouses for neutral fats, and as long as they remain healthy neutral fats alone are present in them. When they are subjected to the action of the pancreatic juice with its fat-splitting ferment the cells are killed and coincidentally the neutral fats are decomposed, fatty acids being deposited. The fatty acids now slowly combine with the calcium salts. In degenerating lipomata the process would seem to be similar.

But in other cases the cells are not obviously fat-containing in the normal state; nevertheless prior to calcification they undergo so-called fatty degeneration, which is really a form of cell degeneration accompanied by fat infiltration. As regards the source of the cell fats in general we may safely accept:

1. That fats are transported in the blood as diffusible soaps.
2. That taken up by the cells these soaps may either—
 - (a) Be reconverted into neutral fats and become stored in the cytoplasm as such, or
 - (b) undergo assimilation proper, becoming part and parcel of the cell substance, in which case they are not recognizable by the ordinary microchemical tests.
3. If these two possibilities be accepted it follows that the appearance of fats and soaps in the degenerating cell may be due to either—
 - (a) Absorption or infiltration of soaps from the surrounding medium, the degenerating cell retaining the power of splitting off the fat but being unable to utilize this in metabolism.
 - (b) Cytoplasmic disintegration with dissociation of the soap-albumen combination or, more broadly, liberation of the fats from their combination with the cytoplasm.

The appearances seen in the cells of atheromatous areas indicate that the first of these does occur.

III. In areas undergoing calcareous infiltration we have demonstrated the presence of soaps, and this often in such quantities that they can be isolated and estimated by gross chemical methods. By microchemical methods also we have been able to show that after removing all the neutral fats and fatty acids by petroleum ether there remains behind a substance giving with Sudan III the reaction we associate with the presence of soap. And experimentally we have produced these soaps within the organism, more particularly by placing capsules containing fats and fatty acids within the tissues and after several days finding that the capsules contain calcium soaps and possess a calcium content far in excess of that of the normal blood and lymph.

IV. While these are the facts, certain of the details of this reaction demand elucidation. The existence of sodium and it may be potassium soaps in the degenerated cells is comprehensible if we accept that these are present in the circulating lymph and simply undergoing absorption. But even then, as these are diffusible substances how is it to be explained that they become stored up in these particular areas?

We have found that, as a matter of fact, in regions which give the reaction for soaps, but which give no reaction for calcium (which therefore presumably contain at most amounts of the insoluble calcium soap too small to need consideration), the ordinary solvents for potassium and sodium soaps do not forthwith remove the stainable material; they are relatively insoluble.

The reason for this insolubility is suggested by the observations made in the test tube, that soap solutions mixed with solutions of white of egg or blood serum form a precipitate of combined soap and albumen, which likewise is insoluble in water and alcohol. The indications are therefore that in cells undergoing degeneration, with degeneration of the cytoplasm, certain albuminous molecules unite with the soaps present to form relatively insoluble soap-albuminate.

V. With regard to calcium soaps, these are also present and in certain stages appear to be the dominating form in the affected tissues. Two questions suggest themselves, viz.: what is the source of calcium, and what is the process by which they become formed?

As to the source, the amount present in well-marked calcification is far in excess of the normal calcium contents of the affected tissue. If in the kidneys of experimental calcification three hundred times as much calcium may be present as in the normal kidney (von Kossa), the calcium must be conveyed to the part by the blood and lymph, and that this is so is demonstrated, as we have pointed out, by the distribution of the infiltration in solid organs, that like ovarian fibroids have undergone necrosis, in which the earliest deposits are superficial.

As to the process, there are three possibilities:

1. That sodium and potassium soaps and soap albuminates are first formed and that interaction occurs between them and the diffused calcium salts from the lymph, the less soluble-calcium replacing the sodium and potassium.

2. That under certain conditions the calcium salts act directly on the neutral fats present in the degenerating cells.

3. That the neutral fats are first broken down into fatty acids and that these react with the calcium salts to form the soaps.

We are assured that the first process occurs and that because in the boundary zone of areas of calcification we can detect soapy particles devoid of calcium, identical in position and arrangement with the particles more deeply placed which give the calcium reactions. But this is not the only reaction. In case of fat necrosis we see clearly that the third process is in evidence. And we are far from being convinced that the second does not also obtain. We have been impressed by the large accumulation of neutral fats in the cells in cases of early atheroma and the absence at any stage of the process of recognizable fatty acid. While soaps, it is true, are compounds of fatty acids with alkalies, it is recognized in ordinary domestic life that they can be formed by the direct action of strong lye upon ordinary fats, and this even in the cold. It is quite possible therefore that there occurs a similar direct process in the organism.

The point is worth noting, however, that this does not occur in healthy cells the seat of fatty infiltration. We therefore leave this an open question, only laying down that, as indicated by

the hyalin albuminous matrix left when calcium salts are dissolved out of an area of calcification, there must exist a calcium soap- or fat-albuminate similar to the potassium and sodium soap-albuminates already mentioned—such an albuminate as we can form with calcium soaps in the test tube.

VI. In old areas of calcification soaps are largely if not entirely wanting, although these are to be detected at the periphery, when the process is still advancing. The reactions given by these older areas are almost entirely those of calcium phosphate, though some calcium carbonate is at times to be made out.

This seems surely to indicate that the final stage in calcification is an interaction between the calcium soap-albuminates and substances containing phosphoric and carbonic acids. Such substances, it is needless to say, are present in considerable amounts in the lymph and blood. We must conclude that the acid sodium phosphates of the lymph act on the calcium soap, the highly insoluble calcium phosphates being formed (plus the albuminous moiety of the original compound) and diffusible sodium soap being liberated, while similarly alkaline carbonates form calcium carbonate and liberate sodium and potassium soaps. Calcium phosphate and calcium carbonate thus become the insoluble earthy salts of old crystalline areas of calcification.

VII. As already stated very little soap is to be found in these old areas. It is possibly worth suggestion that the soaps liberated in this last reaction, as they diffuse out, again react with diffusible calcium salts and form calcium soaps which once more react with the alkaline salts to produce the phosphates and carbonates; that, in short, they have a katalytic action. Certain it is that old calcareous areas are extraordinarily dense, and have a coarse crystalline structure, wholly at variance with the finely granular appearance of the more recent areas, and these large crystalline masses, it would seem, can only be formed by successive deposition of new material and eventual fusion, as the interspaces become filled in between the original masses.

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FIG. 1.

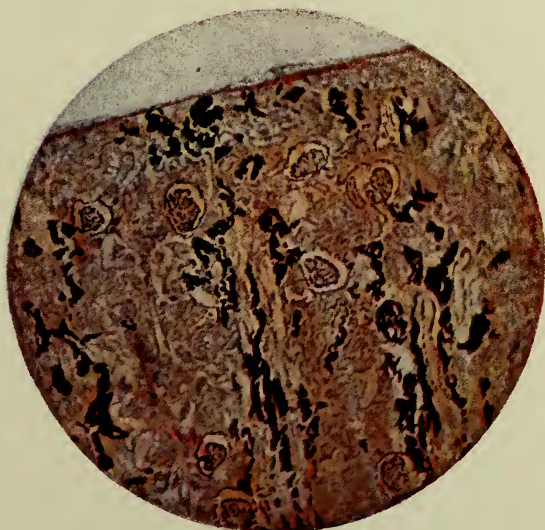


FIG. 2.



FIG. 3.

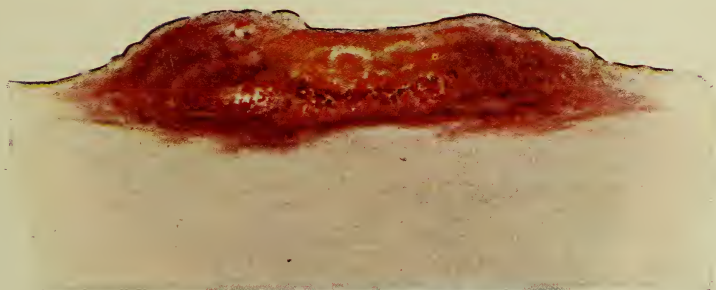


FIG. 4.

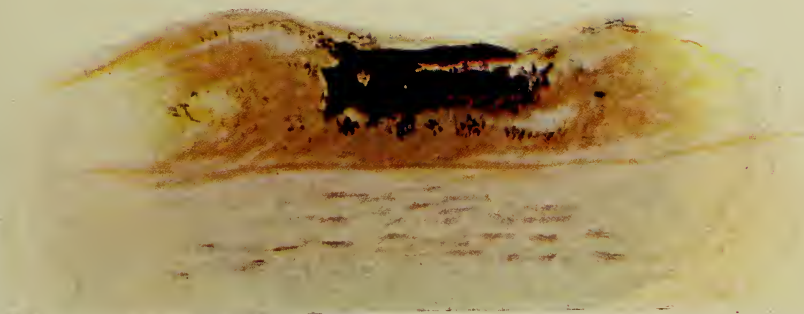


FIG. 5.

DESCRIPTION OF PLATES.

PLATE XXX.

Fig. 1.—Kidney from rabbit which died eight days after inoculation with corrosive sublimate. Frozen section stained with Sudan III showing the fatty condition of certain convoluted tubules.

Fig. 2.—Section from the same kidney as Fig. 1, stained with silver nitrate followed by weak saffranin. Similar convoluted tubules picked out by the silver deposit as seen in Fig. 1 with Sudan III.

PLATE XXXI.

Fig. 3.—Aorta with large fat masses towards the lumen with similar lighter staining particles near the boundary zone between the intima and media. In the media the blackened deposit representing calcium salts is very intense and is arranged in parallel rows. These rows with higher magnification are seen to be composed of numerous small crystals. Among these rows of calcium salts are also seen the yellowish granules of Sudan III staining.

Fig. 4.—Section of aorta stained with Sudan III. The fatty plaque shows a lighter stained part in its middle, representing the area of soap formation and calcium deposit.

Fig. 5.—Same aortic plaque as seen in Fig. 4, treated with silver nitrate solution. The area which with Sudan III stained a light yellow is now represented as the black deposit, the site of the calcium salts.





